Host response to bacterially contaminated titanium and titanium alloy surfaces

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Chapter 6

Cross-talk between immune cells and human mesenchymal stem cells on bacterially contaminated titanium alloy surfaces

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ABSTRACT

We hypothesize that cytokine-mediated cross-talk between immune and stromal cells is influenced by the surface characteristics of the biomaterial surfaces they interact with, the presence of bacterial contamination on these surfaces and the direct contact between the immune and stromal cells. The aim of this study is to develop a co-culture method to measure the cytokine production of host cells *in vitro* in a direct- and indirect-contact mode. In addition, the influence of surface characteristics of biomaterials surfaces and bacterial contamination on cytokine production by the host cells is investigated. This culturing system included primary peripheral blood mononuclear cells (PBMCs), human mesenchymal stromal cells (hMSCs) and heat-killed bacteria or lipoteichoic acid (LTA) to comprehensively mimic the host response to bacterially contaminated titanium alloy surfaces and photocatalytically activated, anodized titanium alloy surfaces. hMSCs, PBMCs and bacterial stimuli were seeded on biomaterial surfaces with direct and indirect cell-cell contact or cell-bacterial stimuli contact in mono-culture and co-culture models. In the mono-cultures, PBMCs produced IL-1β and TNF-α in the presence of bacterial contamination, whilst the hMSCs produced IL-6 and IL-8 but only in the presence of LTA. When these cell types were co-cultured in a direct contact mode with the presence of bacterial stimuli, there was an increase in IL-6 and IL-8 production which was greater than the sum of their corresponding mono-cultures. If the cells co-cultured but hMSCs were separated from PBMCs and bacterial stimuli by a transwell (indirect-contact co-culture), this trend was largely preserved. This suggests that intercellular communication between these cell types is highly important in the development of host responses, though the role of contact-dependent signaling is reduced in comparison to the release of signaling molecules. Furthermore, the results of this work demonstrate the importance of stromal cells in regulating immune responses at the implant site.

Keywords: cytokines; peripheral blood mononuclear cells; human mesenchymal stromal cells; immune responses; titanium alloy.

INTRODUCTION

Biomaterial implants and devices or their implantation sites often become bacterially contaminated during surgical placement. Surgically introduced bacteria can remain dormant on an implant or device surface to cause a biomaterial-associated infection many years later [1, 2]. Despite the relatively high level of wound contamination from sources such as the skin of the patient and surgeon, the operating room air and from any pre-existing wounds, the rates of infection remain relatively low [1]. Why this leads to infection in some cases but not in others is not yet known. However, the biological interactions at a biomaterial surface may play a role. The biomaterial surface interacts with immune and tissue cells to control the inflammatory response and initiate wound healing [3], both in the absence and presence of surgically introduced bacterial contamination. To control wound healing and the associated inflammatory response, host cells first need to be recruited to the implant site, sense the environment and co-ordinate their responses. Communication between the cells occurs through cytokines: small, soluble, signaling proteins secreted by many cell types which bind to cell surface receptors, triggering a series of intracellular signal cascades and eventually modulating target cell behavior and function [4]. The secretion of cytokines by host cells around biomaterial implants and devices can be affected by the characteristics of the biomaterial surface, the cell types present, the status of the cells, cross-talk among different host cells, direct or indirect contact between cells, and the presence of contaminating bacteria [5, 6].

In vivo, wound healing around biomaterial implants always involves multiple cell types like dendritic cells, mast cells, neutrophils, macrophages, mesenchymal stromal cells and fibroblasts [7, 8], possibly combined with the presence of contaminating bacteria. Yet, most *in vitro* studies evaluating host cell behavior on biomaterial surfaces involve only one cell type and cross-talk among different cell types is neglected. One of the few studies involving multiple cell lines demonstrated that co-cultures of osteogenic cells with peripheral blood mononuclear cells (PBMCs) induced a different cytokine pattern specifically increasing interleukin-10 (IL-10), transforming growth factor-β (TGF-β) and decreasing interleukin-1β (IL-1β), compared to the mono-culture of osteogenic cells [9]. The interaction between osteogenic cells and PBMCs, had a larger effect on the proliferation and differentiation of the osteogenic cells than the roughness and surface chemistry of the biomaterials evaluated [9]. This type of direct cell to cell signaling between healing cells and immune cells has also been shown to better mimic the foreign body reaction *in vitro* and to increase cytokine production [10]. Furthermore, bacteria also

play a role in host healing. In a co-culture study between osteoblasts and bacteria [11], it was observed that osteoblast adhesion and spreading were greatly impeded on polyethylene glycol-based surfaces in the presence of contaminating bacteria, while osteoblasts grew and spread well in the absence of bacteria. Adding macrophages to the osteoblast culture increased the survival period of osteoblasts in the presence of highly virulent strains like *Staphylococcus aureus* or *Pseudomonas aeruginosa*. In contrast, in the presence of *Staphylococcus epidermidis*, the presence of macrophages did not change osteoblast adhesion and spreading [12].

There are different methods by which cells communicate at the site of a biomaterial implant and very few models currently used in biomaterial infection research take them into consideration. The first of which is contact-dependent or juxtacrine signaling, which is mediated by direct contact between cells. Secondly there is contact independent or paracrine signaling whereby molecules are released from the cell and can instigate a change in cell behavior over a distance. In this study, we hypothesize that both contact-dependent and indirect cellular signaling between immune cells and stromal cells is an important factor in the behavior of these cells in the presence of a biomaterial. Furthermore, we hypothesize that the presence of bacterial contamination may alter inter-cellular signaling by both signaling mechanisms.

Firstly, we will model the two different types of signaling using a direct-contact co-culture in which immune and healing cells are cultured together at a biomaterial surface and compare this to classical mono-cultures of each cell type. Secondly we will model how stromal and immune cells locally communicate without direct contact. To model this and also to determine the importance of contact-dependent signaling, immune cells will be stimulated with bacterial stimuli and co-cultured with healing cells isolated by a porous membrane. This membrane will prevent the movement of cells whilst allowing the diffusion of signaling molecules thus modeling indirect-contact co-culture. The outcome of the different scenarios will be determined by quantifying the cytokines released by the cultured cells, in the future this will be combined with the ability of the hMSCs to adhere to and survive on the different surfaces to give an indication of healing ability. The cytokines quantified in this study can be seen in Table 1 with a brief description of their sources and major functions.

Table 1. Characteristics of the cytokines studied in this investigation.

Cytokine	Primary sources during inflammation	Major functions
IL-1α	Monocytes, macrophages, keratinocytes, epithelial cells	Broadly inflammatory Stimulates IL-6, IL-7, IL-8 and TNFα Enhances bone formation Accelerates wound healing
IL-1β	Monocytes, macrophages, dendritic cells	Same as IL-1α Stimulates generation of T-helper 17 cells Induces early antimicrobial proteins production
IL-6	Monocytes, T cells, fibroblasts, endothelial cells, keratinocytes, osteoblasts, stromal cells	Mostly inflammatory, also inhibits effects of IL-1 and TNFα whilst stimulating IL-1 receptor antagonist and IL-10 Stimulates osteoclast formation Key role against infection
IL-8	Monocytes, macrophages, neutrophils, lymphocytes, endothelial cells, epithelial cells, fibroblasts, keratinocytes	Inflammatory, primarily neutrophil chemokine and activator Stimulates angiogenesis by stem cells Key role against infection
IL-10	Monocytes, macrophages, B cells, T cells, keratinocytes	Immuno-regulatory Inhibits cytokines including: IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα Enhances humoral immune responses and attenuates cell-mediated immune reactions Inhibits osteogenic differentiation
TNFα	Monocytes, macrophages, B cells, T cells, dendritic cells, fibroblasts	Broadly inflammatory Stimulates IL-1α, IL-1β, Interferon-β, IL-6, IL-8 and TNFα Induces osteolysis Stimulates diapedesis Stimulates phagocytosis

MATERIALS & METHODS

Preparation of photocatalytically activated, anodized titanium alloy surfaces

Commercially available titanium alloy (TAV) discs (Ti6Al4V: grade 5, Salomon's Metalen, Groningen, The Netherlands) with a diameter of 5 mm and a thickness of 1 mm were used in this study. TAV discs were mechanically polished with 1200 grid SiC paper and ultrasonically cleaned in a series of acetone, ethanol and ultra-pure water for 5 min, three times at each step. For anodic oxidation, TAV discs were immersed into 1 M H_2SO_4 solution for 5 min to dissolve their passivation layer. Anodic oxidation of TAV discs was done in the same solution at a constant DC current density of 4 A/cm^2 for 2 min. The cathode was made of TAV (60 mm x 15 mm x 1 mm). After treatment, the anodized discs were extensively rinsed with ultra-pure water and dried in an oven at 40°C for 24 h. Oxidized discs were irradiated with UV light (15 W, wavelength 254 nm) for 5 min in a CleneCab for PCR workstation (CleneCab, Herolab GmbH Laborgeräte, Wiesloch, Germany) at a distance of 60 cm from the UV light tubes (photocatalytic activation). Photocatalytically activated discs were always used within 30 min after UV irradiation and will be denoted as mTAV. Physico-chemical characteristics of thus prepared TAV and mTAV have been described previously [13]. Briefly, TAV has grooves on the surface with surface roughness of 83 ± 9 nm, a water contact angle of 76 ± 1 degrees and no crystalline TiO_2 phases, while mTAV has a porous topography with a surface roughness of 145 ± 21 nm, a water contact angle of 0 degrees and 15% anatase and 85% rutile as crystalline TiO_2 phases.

Isolation of PBMCs from whole blood

Human whole blood was drawn from 3 healthy volunteers using K2-EDTA spray-coated BD Vacutainers® (Becton Dickinson B.V., Breda, The Netherlands) with informed consent, as approved by the Medical Ethical Committee of the University Medical Center Groningen (M13. 137567). PBMCs were isolated using density gradient centrifugation within 30 min after drawing blood from a volunteer. Briefly, 20 ml whole blood from each volunteer was 1 : 1 diluted with sterile phosphate buffered saline (PBS, 137 mM NaCl, 1.47 mM KH_2PO4, 8.1 mM Na_2HPO4 and 2.68 mM KCl, pH 7.0) and added to Leucosep 50 ml tubes (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands) containing 15 ml NycoPrep 1.077. Subsequently, tubes with layered blood samples were centrifuged at 800 × *g* for 15 min at room temperature. Then, the supernatant containing plasma proteins was removed and the pellet containing the PBMCs was transferred into another tube and washed twice with 10 ml sterile PBS at 250 × *g* for 10 min. After washing, cells were resuspended

into minimal essential medium-alpha (α-MEM, Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Breda, The Netherlands) and 0.1 mM ascorbic acid-2-phosphate (Fluka, Steinheim, Germany). The concentration of PBMCs was determined using a Coulter Counter (Beckman Coulter Nederland B.V., Woerden, The Netherlands).

hMSCs culturing and harvesting

Human mesenchymal stromal cells (hMSCs) were isolated from the reaming debris after total hip replacement surgery in a 64-year old female patient (rheumatoid arthritis, not on medication) with informed consent as approved by University Medical Center Groningen (METc2011:143), using Ficoll density gradient centrifugation. The hMSCs obtained were cultured in α-MEM supplemented with 10% heat-inactivated fetal bovine serum, 2% Gibco® antibiotic-antimycotic (10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Fungizone®, Invitrogen, Breda, The Netherlands), and 0.2 mM ascorbic acid-2-phosphate at 37°C in a humidified atmosphere with 5% CO_2. The same batch had previously been characterized by their antigen expression profile (CD73, CD90, CD105, CD34, CD45, CD11b, CD19 and HLA-DR) following the guidelines of the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, and their ability to differentiate in osteogenic, chondrogenic and adipogenic lineages [14]. For the experiments hMSCs were cultured in medium without antibiotic-antimycotic solution. At 80 - 90% confluence, cells were detached using a trypsin-EDTA solution and harvested by centrifugation. Cells were then counted using a Bürker-Türk counting chamber and diluted to the desired concentration (2 x 10^4 cells/ml) in culture medium. hMSCs were used at passage 3 in all the experiments.

Bacterial culturing and heat-killing

Heat-killed bacteria were used as a representative of contaminating bacteria, previously it has been shown the heat-killed bacteria stimulate responses representative of live bacteria [15] and reduce the variability introduced into experiments compared to the use of live bacteria. In addition lipoteichoic acid (LTA, Sigma-Aldrich, Zwijndrecht, The Netherlands) isolated from *S. aureus* was used as a well-defined stimulus for comparison. Briefly, a single colony of *Staphylococcus aureus* ATCC 12600 was inoculated from a blood agar plate in 10 ml tryptone soya broth (OXOID, Basingstoke, UK) and grown aerobically at 37°C for 24 h. Then, 0.5 ml of this culture was added to another 10 ml of fresh tryptone soya broth and incubated for 16 h to form the main-culture. Bacteria from the main-culture were

harvested by centrifugation at 4000 × *g* for 5 min at 10°C, and washed twice with 10 ml sterile PBS. Bacteria were killed by heating the bacterial suspension to 65°C for 1 h. After heat-killing, an aliquot of bacterial suspension was plated on a blood agar plate and incubated overnight at 37°C to confirm complete killing. The number of the heat-killed bacteria was determined by counting in a Bürker-Türk hemocytometer and adjusted to 10^6 bacteria/ml by diluting in sterile PBS.

Culturing of PBMCs and hMSCs on titanium alloy surfaces and quantification of cytokines

Mono- and co-cultures of PBMCs and hMSCs were seeded on TAV or mTAV surfaces placed in 24 well plates within 1 h after isolation in absence or presence of transwell inserts (0.4 µm pore size, Greiner Bio-One B.V.). In mono-culture experiments, 1 ml PBMCs or hMSCs were seeded, while in co-culture, 0.5 ml of each cell type was seeded in one well. In each model 3 stimuli were assessed separately, a suspension of heat-killed *S. aureus* in PBS (10^6 bacteria/ml), a positive control of 10 µl LTA and a negative control of 10 µl PBS alone. In the classical mono-culture experiments, 10 µl of stimulus was added directly in the cell culture. In the transwell isolation experiments the stimulus was added into the transwell to prevent direct contact between bacterial stimuli and hMSCs or PBMCs. In the indirect-contact co-culture the PBMCs together with the stimuli were added above the transwell insert containing a sample disc. Below this hMSCs were seeded onto an additional sample disc in the well plate. An overview of the different experimental conditions can be seen in Figure 1. All of the experiments were performed in 24 well plates incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Following the incubation the media from the wells was centrifuged at 10000 × *g* at 4°C, after which the supernatant of each well was collected and stored at -20°C until the cytokine concentration was measured. For the experiments using a transwell, media was combined from above and below the transwell membrane. Cytokines were detected using a commercially available MILLIPLEX map kit for human cytokine/chemokine magnetic bead panel (Millipore, Billerica, USA) using the Luminex 100 system (Luminex, Austin, USA) according to instructions of the manufacturer.

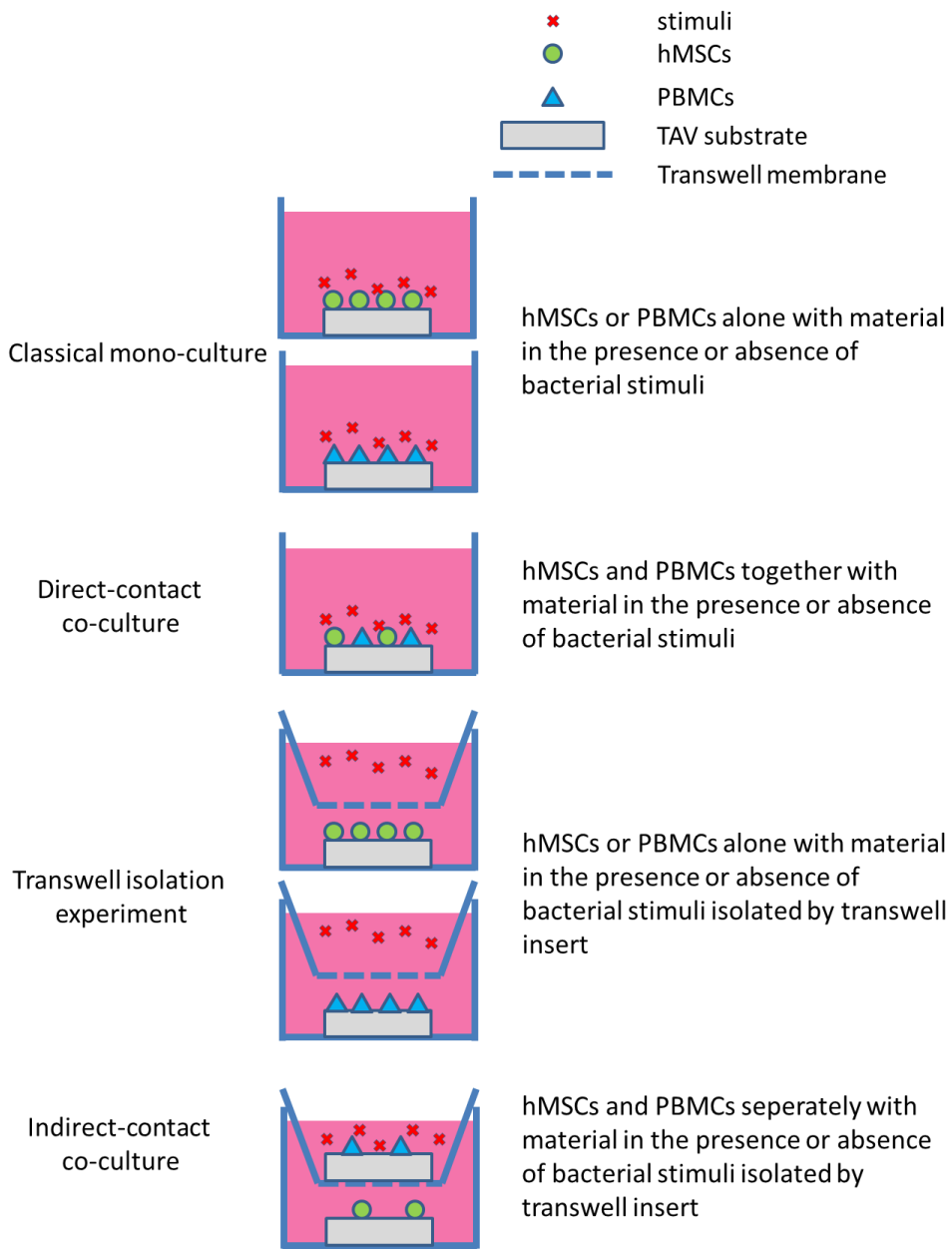

Figure 1: A schematic of the different experimental conditions used in this study.

RESULTS

In the classical mono-culture experiments in absence of bacterial stimuli, hMSCs, produced negligible quantities of both TNF-α and IL-1β (Figure 2). Also PBMCs produced negligible amounts of these cytokines. However, when stimulated by bacterial stimuli both cytokines were produced in greater quantities. In contrast, IL-6 was produced by the hMSCs in all conditions with similar amounts produced in the absence of bacterial stimuli and in the presence of heat-killed *S. aureus*, whilst in the presence of LTA production was greatly increased. The PBMCs produced much smaller quantities of IL-6 for all of the conditions with LTA causing the largest production. IL-8 was produced by hMSCs in large amounts, but only in the presence of LTA. The PBMCs also produced IL-8 in large amounts in the presence of LTA and a lesser amount when stimulated with the heat-killed bacteria. There was no detectable production of IL-1α and IL-10 in both hMSCs and PBMCs mono- and co-cultures, irrespective of the presence of bacterial stimuli (data not shown).

To provide a comparison with the direct-contact model, the average concentration of cytokines produced by each cell type in the mono-cultures was calculated. This is because the co-culture consisted of half the number of each cell type mixed together. This quantity can be seen in Figure 3 as the red lines on each bar. In absence of bacterial stimuli, there was little difference between the direct-contact co-cultures and the average of the classical mono-cultures. For TNF-α and IL-1β the quantities of cytokines produced were also similar to the classical mono-cultures with both bacterial stimuli, though the effect of heat-killed *S. aureus* was slightly increased whilst LTA cytokine secretion was decreased. In contrast IL-6 production was greatly increased in the presence of bacterial stimuli, this was particularly pronounced in the presence of heat-killed *S. aureus*. This same trend was also observed for IL-8 suggesting a synergistic effect caused by co-culturing.

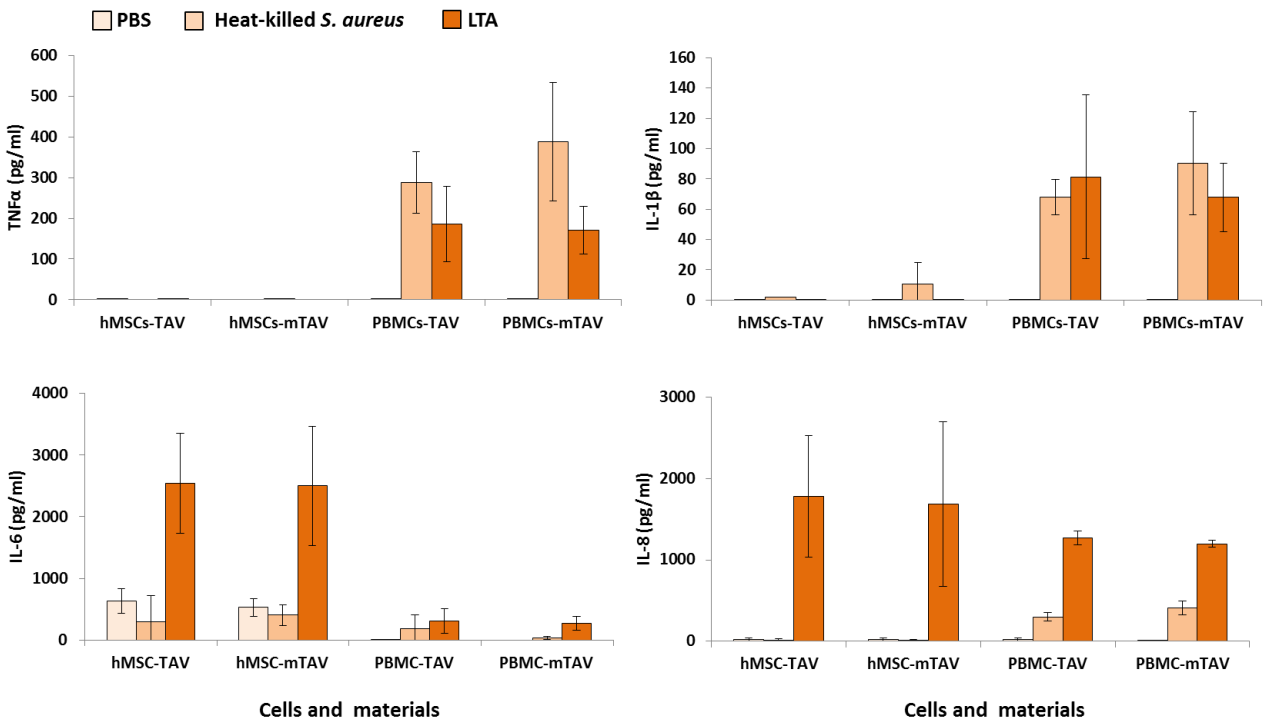

Figure 2. Cytokine production from the classical mono-cultures of hMSCs or PBMCs in the presence of heat-killed *S. aureus* or LTA on TAV and mTAV surfaces after 24 h of incubation. PBS was added as a "negative control" stimulus. Error bars represent standard deviations of two independent experiments

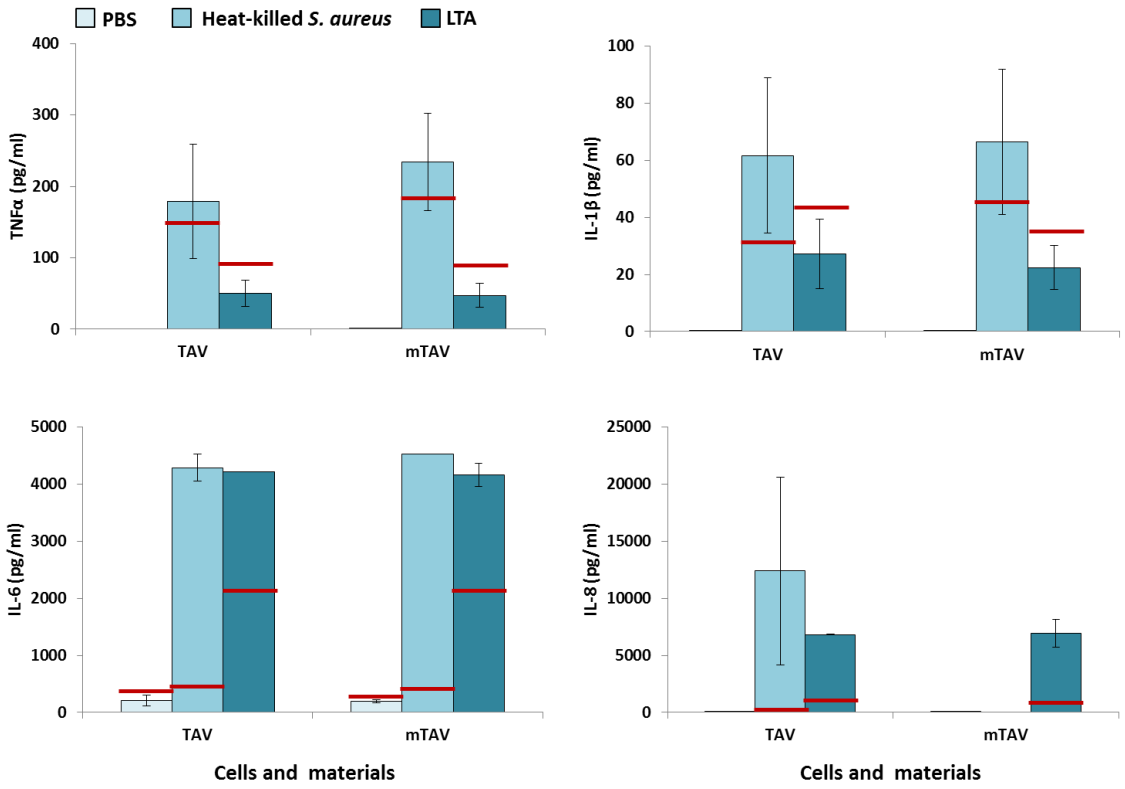

Figure 3. Cytokine production from the co-culture of hMSCs and PBMCs with direct contact with heat-killed *S. aureus* or LTA on TAV and mTAV surfaces after 24 h of incubation (direct-contact co-culture). Error bars represent standard deviations of two independent experiments. Red lines represent the average cytokine production by hMSCs and PBMCs from their corresponding classical mono-cultures. The data for IL-8 on mTAV with *S. aureus* is absent due to a lack of signal in the MILLIPLEX plate.

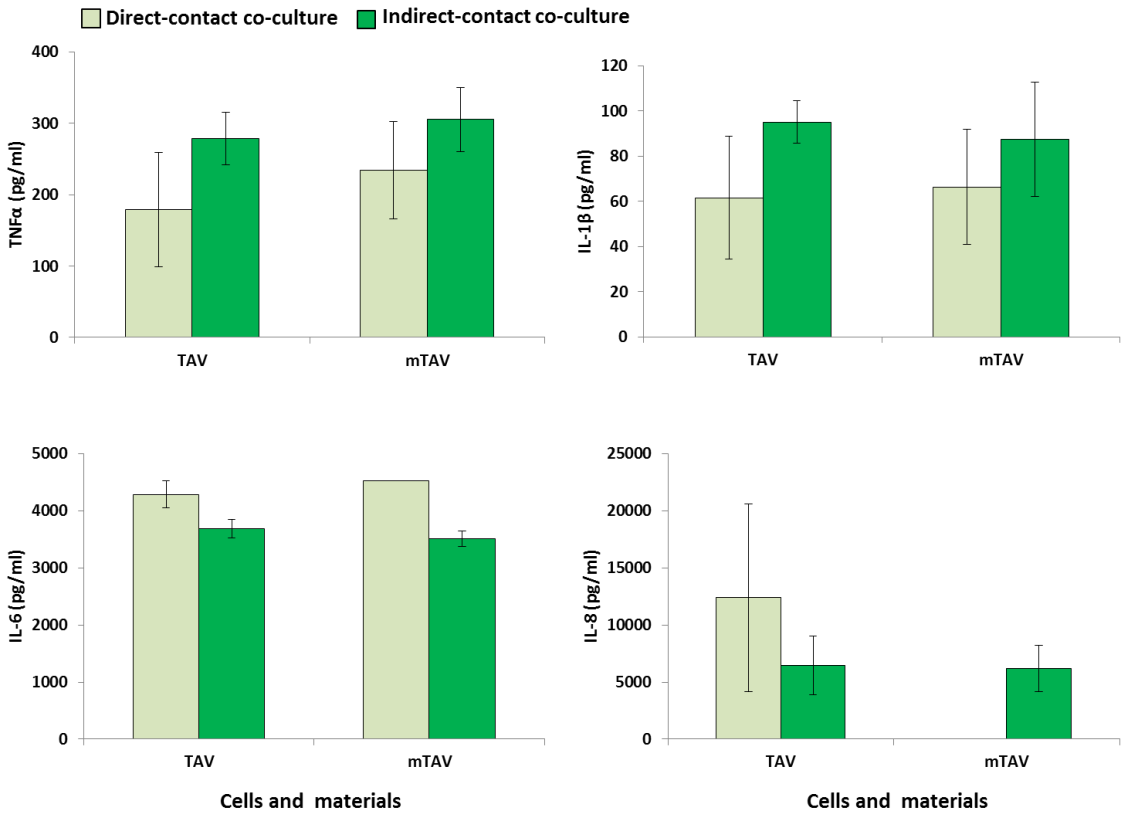

Figure 4. Cytokine production from the co-culture of hMSCs and PBMCs where hMSCs are in direct (direct-contact co-culture) or indirect contact (indirect-contact co-culture) with the PBMCs and the heat-killed *S. aureus* after 24 h incubation. Error bars represent standard deviations of two independent experiments. The data for IL-8 on mTAV with direct-contact co-culture model is absent due to the data reading problem.

In the transwell isolation experiment, the presence of the transwell did not greatly alter the production of cytokines by hMSCs in comparison to the classical mono-cultures (data not shown). IL-6 production was similarly increased in both experiments. Therefore the transwell did not isolate LTA and the condition was excluded from the indirect-contact co-culture experiments. However, when the co-cultures of PBMCs and hMSCs were physically separated by the transwell, TNF-α and IL-1β production was slightly increased returning to values closer to the PBMCs mono-cultures (Figure 4). In contrast, IL-6 and IL-8 production were slightly decreased and still higher than the values of the mono-cultures. Finally, throughout

all of the results in all of the models tested, there was no difference identified between the two materials tested.

DISCUSSION

Immune and tissue cells control the inflammatory response caused by biomaterial implants, both in the absence and presence of bacterial contamination [5]. In any healing process host cells are recruited to the damaged site where they sense the environment, start producing cytokines and also communicate with local cells via direct contact to coordinate responses. In this study we evaluated the role of signaling between hMSCs and PBMCs, considered to play pivotal roles in wound healing after surgical implantation of a biomaterial. We developed a multiple cell culture model in which different situations were studied which may alter the cytokine production of hMSCs or PBMCs including different biomaterial surfaces, bacterial stimuli, co-cultures of hMSCs and PBMCs and direct- or indirect-contact. The interactions between hMSCs and PBMCs cells showed different cytokine profiles depending on mono- or co-cultures and presence or absence of bacterial stimuli. No difference in cytokine production was observed between the two titanium surfaces, TAV and mTAV, that were used. This is an interesting result as previously in Chapter 4, it was observed that modification of TAV in such a way altered cellular adhesion. This did not translate into a change in cytokine production under the conditions tested in this chapter.

The presence of bacterial stimuli in the form of heat-killed *S. aureus* and LTA increased the secretion of cytokines by both hMSCs and PBMCs. Differences in cytokine secretion were also found to be cell type and stimulus dependent. In the classical mono-cultures, PBMCs produced more of the general inflammatory cytokines TNF-α and IL-1β and less of the more specific inflammatory cytokines IL-6 and IL-8 after stimulation. Interestingly however, the presence of LTA stimulated increased production of IL-8 in the PBMCs in comparison to the other situations. This trend was reversed for the hMSC mono-culture with large quantities of IL-6 and IL-8 secreted and none of the other cytokines in quantifiable amounts, but only in the presence of LTA. The upregulation of IL-6 and IL-8 are hallmarks of hMSCs activation via TLRs as observed in other non-biomaterial orientated publications [16]. The production of IL-6 by the hMSCs is of particular interest to biomaterial associated infection studies as this cytokine demonstrates both inflammatory and anti-inflammatory characteristics whilst directing a broadly antibacterial response. IL-6

has the ability to reduce TNF-α and IL-1β production whilst activating IL-10 and IL-1 receptor antagonist. Though we did not see an increase in IL-10 production, a decrease in IL-1β and TNF-α production was observed in the direct-contact co-cultures in the presence of LTA in comparison to the classical mono-cultures suggesting a link. Furthermore, IL-6 is involved in osteoclastogenesis, which may be an important factor in future bone biomaterial research [4]. In the presence of the heat-killed *S. aureus* this trend was not observed suggesting a more complex system. This result may be explained simply by the increased complexity of intact bacteria in comparison to the purified LTA. The reason for this maybe that the hMSCs used have a reduced ability to detect the more complex intact heat-killed bacteria in comparison to the PBMCs. Part of the PBMCs population includes professional immune cells such as monocyte/macrophages, dendritic cells and T-cells which possess a great range of antigen detection molecules. Furthermore, in the direct-contact co-cultures IL-6 and IL-8 production was markedly increased in the presence of bacterial stimuli. The increases in these more specific cytokines suggest an interplay between the cell types to address the presence of infection. This is in agreement with a previous report showing that adding PBMCs to hMSCs with a ratio of (10:1) induced a 6-fold increase in the secretion of IL-6 and IL-8 [17]. In the present study this difference was most obvious with the whole heat-killed bacteria. This strongly indicates that there is a combined response based upon intercellular communication which would not be observed in the standard models of biomaterial associated infection. In the case of the heat-killed bacteria, it may be that the PBMCs detect the bacterial stimuli more readily than the hMSCs alone and begin to produce IL-1β. IL-1β in turn stimulates the production of IL-6 and IL-8 in the stromal cells with more potency than the bacterial stimuli itself. In this way the immune cells act as a detection mechanism and the stromal cells act as a manufacturing plant for the more specific response molecules. It is known that hMSCs cooperate with immune cells at the inflammation sites, the secretion of proinflammatory cytokines like TNFα and IFN-γ is inhibited and secretion of suppressive cytokines like IL-10 enhanced [17]. However this was not observed in our study. The reduction in IL-1β observed in the present study is in agreement with the immunomodulatory role of hMSCs previously seen with macrophages. When in contact with lipopolysaccharide activated macrophages, hMSCs release STC-1 which negatively regulates NLRP3 inflammasome activation and reduces IL-1β secretion a reactive oxygen species generation in these cells [18]. By altering the cytokine production profile of immune cells, hMSCs are capable of modulating the host immune responses to foreign bodies including both implants and contaminating microorganisms. In the future it would be interesting to

test a broader range of cytokines, including those specific to antibacterial responses such as IL-17A. Furthermore, the way in which these interactions affect the ability of the hMSCs to adhere to the surface is of great interest. This is particularly important for the mTAV surfaces which have previously demonstrated the ability to kill bacteria upon contact. The effect these dead bacteria may have on the interplay between the immune cells and the healing cells can be further investigated using the model presented in this Chapter.

The influence of biomaterial characteristics on cytokine production by host cells can give direction to new developments in biomaterials with preferable surface characteristics. The influence of bacterial stimuli on the cytokine production of host cells can be a signal for a biomaterial-associated infection. The co-culture of hMSCs and PBMCs shows the interplay between different cell types and how this plays a role in the integration of the implant. Such a model including primary hMSCs, PBMCs, bacterial stimuli and different biomaterials may better predict the *in vivo* behavior of biomaterials in a more comprehensive way in comparison to existing culture systems thus guiding better future biomaterial development.

CONCLUSIONS

In conclusion, we developed a model of cellular interactions at the biomaterial implant site with and without bacterial contamination. Using this model we observed that cellular signaling was strongly influenced by the ability of cells to communicate and the presence or absence of bacterial stimuli. The results of this work demonstrate that both contact-dependent signaling and communication via released molecules between healing and immune cells are important parts of the host response and should not be overlooked in future biomaterial studies.

REFERENCES

1. Gurkan I, Wenz JF. Perioperative infection control: an update for patient safety in orthopedic surgery. Orthopedics 2006;29:329-39.
2. Zimmerli W, Trampuz A. Biomaterial-associated infection: A perspective from the clinic. In: Moriarty TF, Zaat SAJ, Busscher HJ, editors. Biomaterial-associated infection-immunological aspects and antimicrobial strategies, New York Heidelberg Dordrecht London: Springer, 2013. p. 3-25.
3. Schutte RJ, Xie L, Klitzman B, Reichert WM. In vivo cytokine-associated responses to biomaterials. Biomaterials 2009;30:160-8.
4. Hughes FJ, Turner W, Belibasakis G, Martuscelli G. Effects of growth factors and cytokines on osteoblast differentiation. Periodontology 2000 2006;41: 48-72.
5. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. Semin Immunol 2008;20: 86-100.
6. Nowzari H, Botero JE, DeGiacomo M, Villacres MC, Rich SK. Microbiology and cytokine levels around healthy dental implants and teeth. Clin Implant Dent Relat Res 2008; 10:166-73.
7. Williams DF. Tissue-biomaterial interactions. J Mater Sci 1987;22:3421-45.
8. Anderson JM. Biological responses to materials. Annu Rev Mater Res 2001;31: 81-110.
9. Moura CG, Souza MA, Kohal RJ, Dechichi P, Zanetta-Barbosa D, Jimbo R et al. Evaluation of osteogenic cell culture and osteogenic/peripheral blood mononuclear human cell co-culture on modified titanium surfaces. Biomed Mater 2013;8:035002.
10. Holt DJ, Chamberlain LM, Grainger DW. Cell–cell signaling in co-cultures of macrophages and fibroblasts. Biomaterials 2010;31:9382-94.
11. Saldarriaga Fernandez IC, Busscher HJ, Metzger SW, Grainger DW, Van der Mei HC. Competitive time- and density-dependent adhesion of staphylococci and osteoblasts on crosslinked poly(ethylene glycol)-based polymer coatings in co-culture flow chambers. Biomaterials 2011;32:979-84.
12. Subbiahdoss G, Saldarriaga Fernández IC, da Silva Domingues JF, Kuijer R, Van der Mei HC, Busscher HJ. In vitro interactions between bacteria, osteoblast-like cells and macrophages in the pathogenesis of biomaterial-associated infections. PloS One 2011;6:e24827.
13. Yue C, Kuijer R, Kaper HJ, Van der Mei HC, Busscher HJ. Simultaneous interaction of bacteria and tissue cells with photocatalytically activated, anodized titanium surfaces. Biomaterials 2014;35:2580-7.
14. Buizer AT, Veldhuizen AG, Bulstra SK, Kuijer R. Static versus vacuum cell seeding

on high and low porosity ceramic scaffolds. J Biomater Appl 2013;29:3-13.
15. Li N, Russell WM, Douglas-Escobar M, Hauser N, Lopez M, Neu J. Live and heat-killed Lactobacillus rhamnosus GG: effects on proinflammatory and anti-inflammatory cytokines/chemokines in gastrostomy-fed infant rats. Pediatr Res 2009; 66:203-7.
16. Tomchuck SL, Zwezdaryk KJ, Coffelt SB, Waterman RS, Danka ES, Scandurro AB. Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. Stem cells 2008;26: 99-107.
17. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-22.
18. Oh JY, Ko JH, Lee HJ, Yu JM, Choi H, Kim MK et al. Mesenchymal stem/stromal cells inhibit the NLRP3 inflammasome by decreasing mitochondrial reactive oxygen species. Stem Cells 2014;32:1553-63.